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Bypassing CFTR dysfunction in cystic fibrosis with alternative pathways for anion transport

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ABSTRACT

One therapeutic strategy for cystic fibrosis (CF) seeks to restore anion transport to affected epithelia by targeting other apical membrane Cl⁻ channels to bypass dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel. The properties and regulation of the Ca²⁺-activated Cl⁻ channel TMEM16A argue that long-acting small molecules which target directly TMEM16A are required to overcome CFTR loss. Through genetic studies of lung diseases, SLC26A9, a member of the solute carrier 26 family of anion transporters, has emerged as a promising target to bypass CFTR dysfunction. An alternative strategy to circumvent CFTR dysfunction is to deliver to CF epithelia artificial anion transporters that shuttle Cl⁻ across the apical membrane. Recently, powerful, non-toxic, biologically-active artificial anion transporters have emerged.

HIGHLIGHTS

- Cl^- channels distinct from CFTR are found in the apical membrane of CF epithelia.
- TMEM16A and SLC26A9 are promising targets to bypass CFTR dysfunction in CF.
- Long-acting drugs that target directly TMEM16A are required to mimic CFTR activity.
- SLC26A9, a CF disease modifier, supports sustained Cl^- secretion by epithelia.
- Biologically-active artificial anion transporters have been identified.

INTRODUCTION

The genetic disease cystic fibrosis (CF) is caused by mutations in the epithelial anion channel cystic fibrosis transmembrane conductance regulator (CFTR) [1,2], which plays a pivotal role in transepithelial ion transport [3]. Because of the large number of disease-causing mutations in the *CFTR* gene (<http://www.genet.sickkids.on.ca/app>) and their complex effects on CFTR expression and function [4], there is significant interest in therapeutic strategies which are mutation-independent.

One mutation-independent therapeutic strategy is to exploit other pathways for anion transport in the apical membrane of CF epithelia to rehydrate epithelial surfaces lining ducts and tubes (Figure 1). Most work in this area has focused on the Ca^{2+} -activated Cl^- channel and has included clinical trials of long-acting P2Y_2 receptor agonists that act indirectly by increasing the intracellular free Ca^{2+} concentration [5,6]. However, targeting apical membrane Cl^- channels is not the only possible approach. First, inhibition of the epithelial Na^+ channel (ENaC) prevents dehydration of CF airway epithelia by ENaC-mediated Na^+ absorption [7,8]. Second, activation of basolateral membrane K^+ channels enhances the driving force for Cl^- exit across the apical membrane of CF epithelia through either residual CFTR Cl^- channels or other apical membrane Cl^- channels [9,10]. Third, inhibition of the nongastric H^+/K^+ -ATPase (ATP12A) prevents deleterious acidification of airway surface liquid (ASL), which promotes bacterial growth [11*]. Finally, artificial anion channels and transporters might be delivered to CF epithelia to restore transepithelial Cl^- transport [12] (Figure 1). Here, we examine the utility of targeting apical membrane Cl^- channels to bypass CFTR dysfunction in CF and discuss recent progress in the development of artificial anion transporters.

TMEM16A: A TARGET TO STIMULATE ANION TRANSPORT BY AIRWAY EPITHELIA

TMEM16A (or anoctamin-1 [ANO1]) is the molecular identity of the Ca^{2+} -activated Cl^- channel [13-15]. It mediates the bidirectional flux of Cl^- , HCO_3^- and other anions across the apical membrane of epithelia [5]. When compared to CFTR [2], the structure, function and regulation of TMEM16A differ noticeably. Structural information from a distant paralog, the fungus *Nectria haematococca* [16], argue that TMEM16A is a homodimer with each subunit containing at least ten membrane-spanning helices and a Ca^{2+} -binding site. Intriguingly, the TMEM16A pore might only in part be delimited by transmembrane helices, the remainder being formed in some way by lipids of the plasma membrane [17*]. This unconventional pore structure arises from the lipid scramblase function of distant paralogs [16]. A cleft on each side of the homodimer forms a pathway through which the heads of phospholipids can translocate from one side of the membrane to the other. Although scramblase activity has been conserved in some family members, such as TMEM16F [18], in TMEM16A and TMEM16B, the pathway for lipid head translocation has evolved into a pore for anions.

TMEM16A is expressed in the airway epithelium, although its localisation appears different to that of CFTR. In the surface epithelium, CFTR is predominantly expressed in ciliated cells, whereas TMEM16A is more abundant in mucus-secreting goblet cells [19,20]. Like CFTR, TMEM16A is also present in submucosal glands of the bronchi [21]. Interestingly, TMEM16A expression is increased under pro-inflammatory conditions (e.g. treatment with cytokines or bacterial components), which lead to mucus hypersecretion [19,21,22].

Physiologically, TMEM16A is activated by stimuli that mobilise intracellular Ca^{2+} , such as purinergic agonists. In the surface epithelium, this mechanism might link release of

intracellular ATP [23], caused by mechanical stress (e.g. cough) or other stimuli, to activation of TMEM16A-dependent anion secretion. As revealed by short-circuit current recordings (one measure of transepithelial ion transport), Ca^{2+} -dependent secretion is usually characterised by a large transient, attained a few seconds after stimulation, which declines to near resting levels within 15-20 minutes [5] (Figure 2). This behaviour suggests that TMEM16A is involved in rapid adaptation to conditions where increased anion secretion is required. Given its localisation in goblet cells, TMEM16A might play an important role mediating bicarbonate secretion to support mucus release. By contrast, CFTR function has a more “tonic” role. Its activity is maintained for prolonged periods [3] (Figure 2), suggesting that CFTR contributes to basal anion secretion.

Nevertheless, TMEM16A might represent an alternative therapeutic target to circumvent CFTR dysfunction in the airway epithelia of CF patients. Pharmacological stimulation of TMEM16A function could help to normalise the properties of airway surface liquid and mucus by increasing chloride and bicarbonate secretion. For example, TMEM16A stimulation might be used as an adjuvant therapy, in combination with CFTR modulators that rescue the apical membrane expression and function of CF mutants. It might be especially valuable to individuals carrying CF mutations unresponsive to CFTR modulator therapy.

An ideal drug stimulating TMEM16A should act directly upon TMEM16A, not work through elevation of intracellular Ca^{2+} [24]. It should also produce long-lasting activation of TMEM16A to mimic the normal behaviour of CFTR. Finally, this drug should be delivered by nebulization to avoid systemic administration, which might elicit unnecessary and potentially harmful TMEM16A activation in other organs and tissues, but it also should avoid activation of TMEM16A in bronchial smooth muscle to prevent bronchial constriction [5]. Of note,

Danahay et al. (Poster 91, 14th European Cystic Fibrosis Society Basic Science Conference, Albufeira, Portugal, March 2017) reported the identification of chemical structures that potentiate TMEM16A independent of elevation of intracellular Ca^{2+} . It is also feasible that TMEM16A activators and potentiators might be identified among the chemical structures of TMEM16A inhibitors identified by high throughput screening [5,24]. Lessons from the development of CFTR modulators will no doubt accelerate work to transform small molecule TMEM16A modulators into therapeutics for CF lung disease.

SLC26A9: AN ALTERNATIVE TARGET TO BYPASS CFTR DYSFUNCTION IN CF

One member of the solute carrier 26 (SLC26) family of anion transporters, SLC26A9 has recently been identified as a promising target to bypass CFTR dysfunction in CF epithelia [25,26]. While most SLC26 proteins function as anion transporters, SLC26A9 is a Cl^- -selective channel with limited HCO_3^- conductance [25,27]. Like CFTR, the SLC26A9 Cl^- channel has a linear current-voltage (I-V) relationship with a low single-channel conductance [25,28,29]. However, in marked contrast to CFTR and TMEM16A, which are tightly regulated by cAMP-dependent phosphorylation and the intracellular free Ca^{2+} concentration, respectively [2,5], SLC26A9 is constitutively active, supporting sustained Cl^- secretion once inserted into the apical membrane of airway epithelia [25,26,29,30] (Figure 2). These data argue that SLC26A9 is an important target for mutation-independent therapy in CF.

The predicted topology of SLC26A9 contains a PDZ-binding motif at its C-terminus through which SLC26A9 can interact with CFTR, if both proteins are expressed in the same cell [31-33*]. Consistent with this idea, co-expression of SLC26A9 with wild-type CFTR increased the function of both Cl^- channels, whereas co-expression of SLC26A9 with the CF mutant F508del-CFTR, which fails to reach the plasma membrane, reduced SLC26A9 function

[25,33*,34]. These reciprocal interactions of CFTR and SLC26A9 are likely relevant for the pathophysiology and pharmacotherapy of CF lung disease. First, inhibition of constitutive SLC26A9 activity in airway epithelia by F508del-CFTR might abrogate an important fail-safe mechanism for Cl⁻ secretion, thus aggravating the dehydration of epithelial surfaces in CF patients carrying the F508del-CFTR mutation. Conversely, SLC26A9 might augment the residual function and pharmacological rescue of CF mutants present at the apical membrane (e.g. the gating mutation G551D-CFTR). Consistent with these ideas, Strug *et al.* [35*] found an association between a single nucleotide polymorphism in the *SLC26A9* gene and the magnitude of treatment response in CF patients with gating mutations treated with the CFTR potentiator ivacaftor. The authors also found improved transepithelial Cl⁻ transport in F508del homozygous primary bronchial epithelial cultures treated with the CFTR corrector lumacaftor. These and other data [36] argue that SLC26A9 acts as a modifier of CF lung disease severity and the response to CFTR modulator therapy.

Further evidence supporting SLC26A9 as a promising therapeutic target for mucobstructive lung diseases was obtained using SLC26A9-deficient mice challenged with intrapulmonary installation of the Th2 cytokine IL-13 to induce goblet cell metaplasia and mucus hypersecretion [30]. Like TMEM16A [13], SLC26A9 function is induced by IL-13 treatment [30]. In wild-type mice treated with IL-13, SLC26A9-mediated Cl⁻ secretion prevented airway mucus obstruction despite substantial mucus hypersecretion, whereas in SLC26A9-deficient mice treated with IL-13, which lacked this Cl⁻ secretory capacity, severe airway mucus plugging developed [30]. Interestingly, studies of CFTR-deficient mice revealed that concomitant absence of SLC26A9 causes a substantial increase in gastrointestinal tract-related mortality, indicating that SLC26A9 is a genetic modifier of meconium ileus-like intestinal obstruction in CF mice [37]. Consistent with this idea, SLC26A9 polymorphisms

have also been associated with the risk to develop meconium ileus and early exocrine pancreatic disease in CF patients [38,39].

In summary, SLC26A9 has emerged as a promising therapeutic target to bypass CFTR dysfunction in the respiratory airways, gastrointestinal tract and pancreas of CF patients. However, current knowledge of the SLC26A9 Cl⁻ channel is limited. Considerable research is therefore required before SLC26A9 can be tested as a therapeutic target in patients with CF and potentially other muco-obstructive lung diseases. The crystal structure of an SLC26 homolog from the bacterium *Deinococcus geothermalis*, a proton-driven fumarate transporter [40] should accelerate progress towards this goal.

BIOLOGICALLY-ACTIVE ARTIFICIAL ANION TRANSPORTERS

An alternative approach to CFTR bypass therapy is to create artificial pathways for anion transport that can be inserted into the apical membrane of CF epithelia to mediate transepithelial Cl⁻ transport. Transmembrane Cl⁻ transport might be achieved using static pathways for anion movement (synthetic anion channels) or small molecules, which shuttle anions across cells membranes (artificial anion transporters or anionophores) (Figure 1). In an early proof of principle study, Wallace *et al.* [41] demonstrated that a synthetic peptide derived from the glycine receptor induced sustained transepithelial Cl⁻ secretion across cultured renal epithelia. Subsequently, self-assembled non-peptide synthetic anion channels have been developed and their biological activity assessed [12,42]. Of note, Jiang *et al.* [43] demonstrated that a squalamine analogue, functioning as a synthetic Cl⁻ channel, partially corrected loss of CFTR function in CF airway cells, epithelia and the nasal epithelium of a CF mouse model. Subsequently, Shen *et al.* [44] showed that a synthetic Cl⁻ channel based on a C₂-symmetric isophthalamide scaffold conferred a Cl⁻ conductance on CF airway epithelial cells, while a

modified C₂-symmetric isophthalamide scaffold has been identified, which transports Cl⁻ and HCO₃⁻ across synthetic lipid vesicles and airway epithelia and confers Cl⁻ transport on CF airway epithelia [45].

The successful development of biologically-active anionophores depends greatly on their chemical structure. This chemical structure should bind anions at the interface between the aqueous extracellular environment and the plasma membrane, translocate them across the lipid bilayer before release on the intracellular side (Figure 1). Anionophores therefore require solubility within the lipid bilayer, while forming a binding site for anions shielded from the lipid bilayer. These characteristics are well illustrated by cholapods, steroid-based scaffolds derived from cholic acid [46-48]. The lipophilic nature of cholic acid promotes solubility in lipid membranes, while its rigid structure has well-spaced side groups that can be modified to form a high-affinity Cl⁻ binding site (for review, see [48]) (Figure 3). When tested in synthetic lipid vesicles, cholapods proved to be powerful anion transporters, some exhibiting anion transport at cholapod to lipid ratios as low as 1:250,000 (i.e. one or two anionophores per lipid vesicle) [47,48]. The observation that the central part of the cholapod structure contained most of its anion transport activity [48] motivated the development of a second family of anionophores, termed decalins [49] (Figure 3). Of note, decalins have improved drug-like properties, including smaller molecular weight and reduced lipophilicity, making them easier to partition from aqueous to lipid environments. Moreover, some decalins achieve impressive transport rates in synthetic lipid vesicles, approaching that of CFTR in natural membranes [50].

Using cells engineered to express the halide-sensitive fluorophore YFP-H148Q/I152L [51], Li *et al.* [52*] tested the biological activity of 15 anionophores representative of different chemical scaffolds, including cholapods [46,47], decalins [49,50] and cyclohexanes [53]. One

decalin, a bis-ureiododecalin demonstrated especially promising activity, including (i) delivery to cell membranes from the aqueous solution, (ii) potency at low micromolar concentrations, (iii) sustained activity, transporting anions for up to 2 hours and (iv) lack of cytotoxic effects when tested on three different epithelial cell lines [52*]. These data argue that anionophores are worthy of further investigation as a CFTR bypass therapy for CF. However, important challenges remain to be addressed, including optimisation of chemical structure, regulation and delivery to airway epithelia *in vivo*. Encouragingly, recent data argue that anionophores with encapsulated Cl⁻ binding sites will likely achieve transmembrane Cl⁻ transport with high Cl⁻ selectivity [54*]. Given the importance of HCO₃⁻ for epithelial physiology (e.g. [11*,55]), future studies should also seek to develop anionophores capable of transmembrane HCO₃⁻ transport.

CONCLUSIONS

Mutation-independent therapies that circumvent CFTR dysfunction have a key role to play in the armamentarium of innovative new therapies that tackle the root cause of CF. These therapies are likely to be particularly useful for individuals carrying mutations that are not amenable to treatment with small molecule CFTR modulators. The molecular identification of TMEM16A has accelerated the search for long-acting small molecule activators and potentiators of the epithelial Ca²⁺-activated Cl⁻ channel to achieve sustained transepithelial Cl⁻ secretion. SLC26A9 is an attractive target to circumvent CFTR dysfunction in CF, but much remains to be learnt about this epithelial Cl⁻ channel before therapeutically-active small molecule SLC26A9 activators and potentiators are developed. Similarly, work to develop therapeutically-active anionophores is at an early stage, but the identification of powerful, non-toxic biologically-active anionophores augurs well for the future. Evaluation of CFTR bypass strategies using animal models (ferrets and pigs) resembling CF disease in humans and

organotypic cell cultures will be important for their development as therapeutic tools for CF lung disease.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

Figure 1: Pharmacological rescue of epithelial Cl⁻ transport in CF by targeting alternative Cl⁻ channels or using artificial anion channels and transporters The schematic shows a thin layer of airway surface liquid (ASL) covering the airway surface epithelium and a submucosal gland. The activity of CFTR and the epithelial Na⁺ channel (ENaC) in the apical membrane controls the quantity and composition of ASL. Submucosal glands deliver mucus and antimicrobial factors to the ASL. The insets show the cellular mechanism of CFTR-dependent Cl⁻ secretion and mechanisms to bypass CFTR dysfunction in CF: activation of TMEM16A and SLC26A9, Cl⁻ channels endogenously expressed in the apical membrane of goblet and ciliated cells of the airway epithelia and artificial anion channels and transporters that might be delivered to airway epithelia by nebulisation. Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na⁺ channel; SLC26A9, solute carrier 26 member A9; TMEM16A, transmembrane member 16A. For further information, see the text.

Figure 2: Time course of Cl⁻ current mediated by CFTR, TMEM16A, SLC26A9 and a bis-ureiododecalin anionophore (A – E) Time courses of transepithelial Cl⁻ current recorded with the Ussing chamber technique. The recordings are from human bronchial epithelia endogenously expressing CFTR and TMEM16A (A, B); Fischer rat thyroid (FRT) epithelia expressing recombinant SLC26A9 and control transfected epithelia (C); Madin Darby canine (MDCK) epithelia endogenously expressing canine CFTR (D) and FRT epithelia treated with a bis-ureiododecalin anionophore (2 μM) or the vehicle POPC (20 μM) (E). All recordings were made in the presence of a Cl⁻ concentration gradient with [Cl⁻]_{basolateral} > [Cl⁻]_{apical}. In A-C, the epithelium was intact, whereas in D and E, the basolateral membrane was permeabilised with nystatin (0.36 mg ml⁻¹; Sigma-Aldrich). Arrows indicate the addition of the cAMP

agonists CPT-cAMP (100 μ M) (A), forskolin (Fsk; 1 μ M) with 3-isobutyl-1-methylxanthine (IBMX; 100 μ M) (C) and forskolin (10 μ M) (D); the P2Y₂ agonist UTP (100 μ M) (B) and the start of the recording (C). Recordings were ended without using inhibitors to return current to baseline except A and E, where this was achieved using CFTR_{inh}-172 (I172; 10 μ M) and by removing the [Cl⁻] gradient (E); dashed lines indicate the basal current level. For further information, see the text. Reproduced, with permission, from Salomon *et al.* [29] and Li *et al.* [50*].

Figure 3: Cholapod and decalin anionophores The chemical structures of some biologically-active cholapods (where R = CF₃ and NO₂; X = OCH₃) and decalins (where R = CF₃ and NO₂; X = OC₂H₅) are shown together with the crystal structure of the bis-ureiododecalin with Me₄N⁺Cl⁻. In the crystal structure, the dashed lines denote hydrogen bonds that bind the Cl⁻ ion; the Me₄N⁺ cation is shown in pink. Modified, with permission, from Li *et al.* [50*].

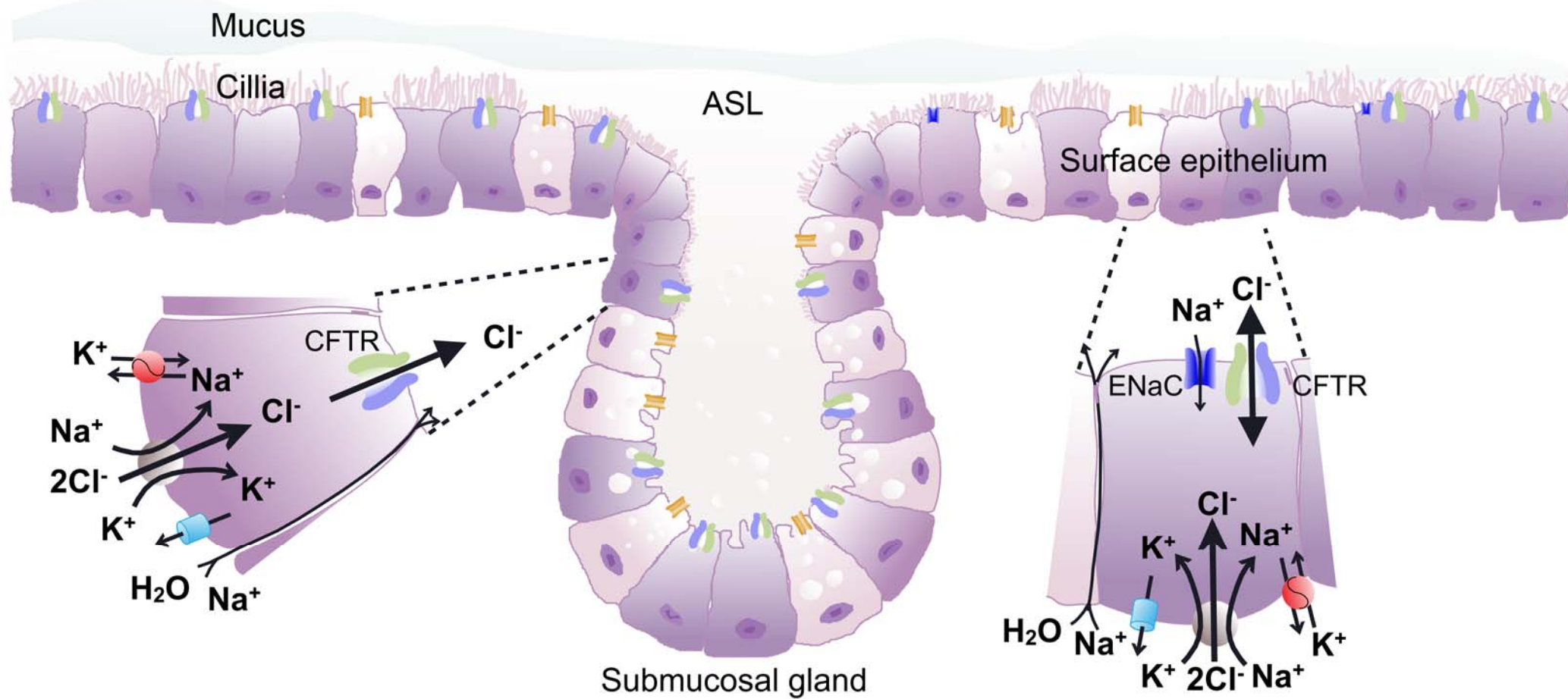
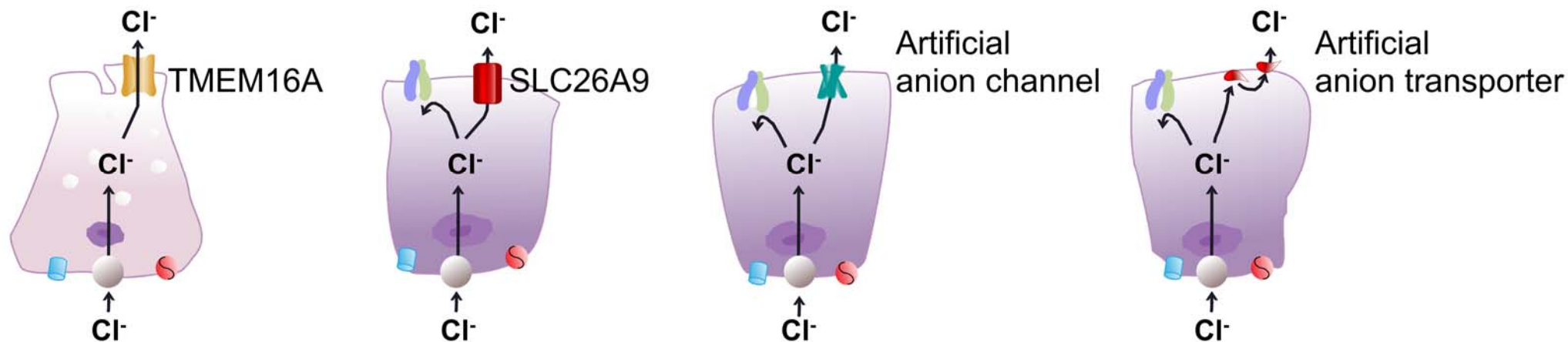


Figure 2

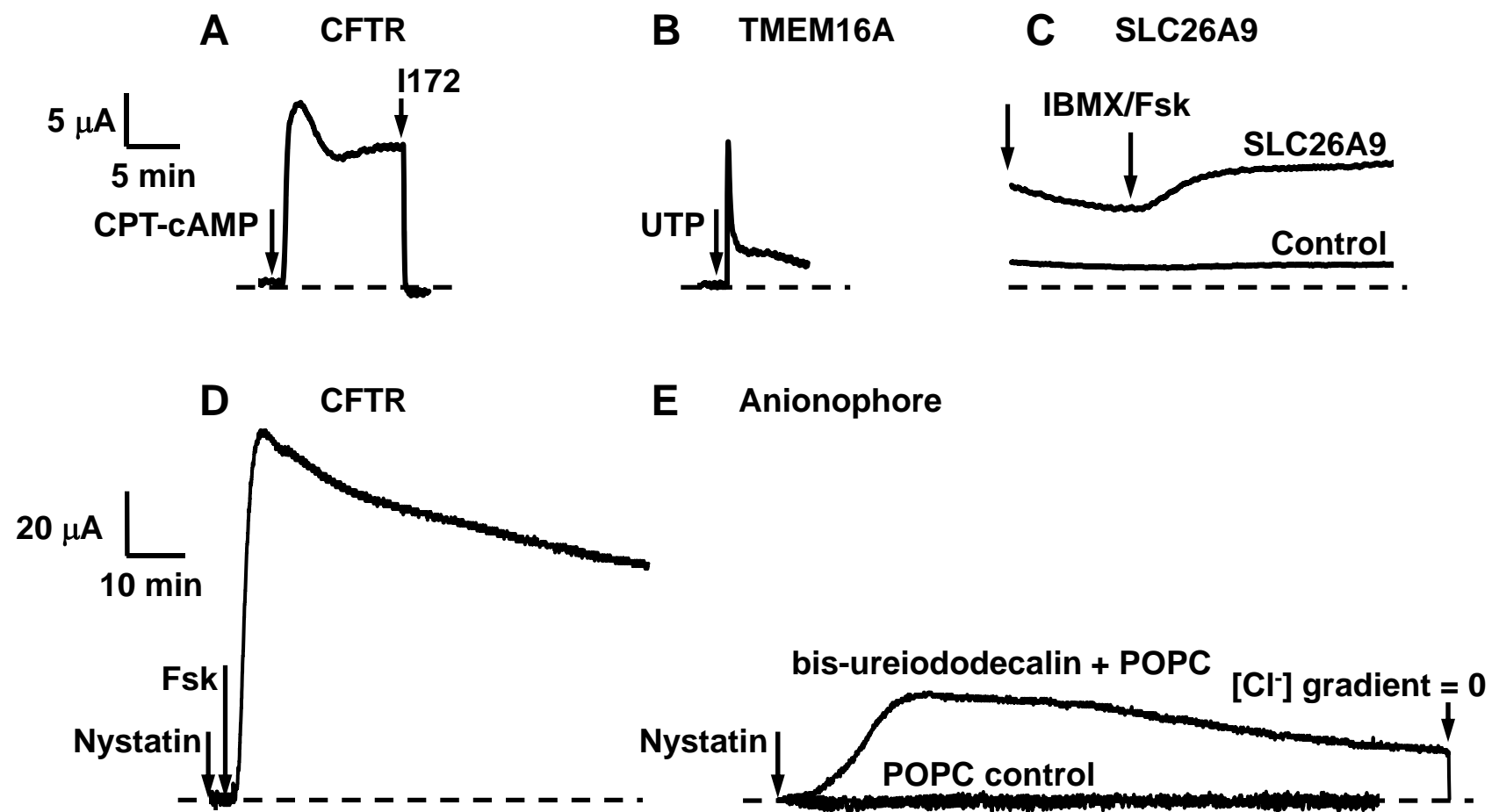
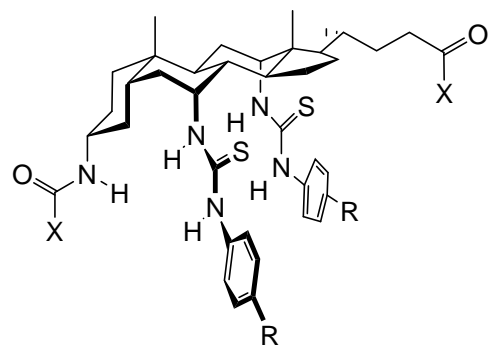
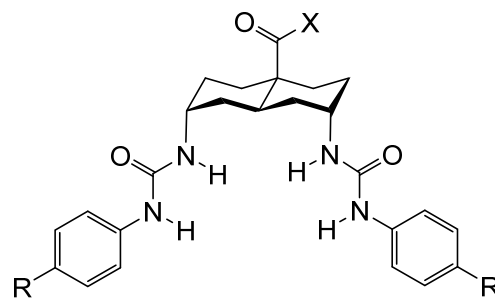


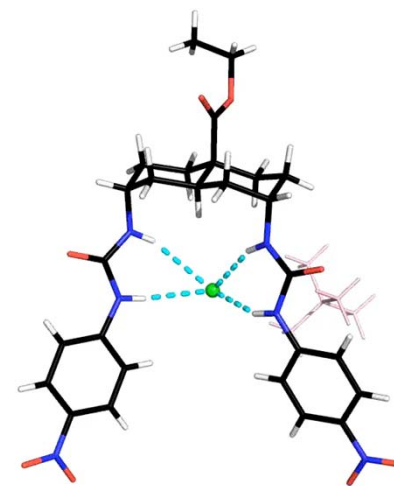
Figure 3



Cholapod



Decalin



bis-ureidodecalin + Me₄N⁺Cl⁻